Gastrulation defective, a complement factor C2/B-like protease, interprets a ventral prepatter in *Drosophila*

Robert DeLotto⁺

Genetics Department, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark

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gastrulation defective (*gd*) encodes a serine protease required for specification of dorsal-ventral cell fates during *Drosophila* embryogenesis. Using RNA microinjection, I show that wild-type *gd* RNA can restore ventrolateral pattern elements with correct polarity with respect to egg shape in embryos lacking *gd* function. While low RNA concentrations restore ventrolateral pattern elements, higher concentrations ventralize the embryo. Gastrulation defective concentration has a rate-limiting effect on the domain of high Dorsal concentration but little effect upon the slope of the gradient. In embryos from *pipe*-null females, much higher RNA concentrations generate an ectopic axis oriented with respect to the site of injection. The data suggest that the Dorsal gradient is not directly determined by asymmetric cues in the eggshell but arises *de novo* within the perivitelline space as a consequence of self-regulatory properties of the protease cascade. A homology to the mammalian complement factors C2 and B is also described.

INTRODUCTION

Cell fates along the dorsal-ventral (d-v) axis of the *Drosophila* embryo are established by a signal transduction pathway directing the production of the Dorsal morphogen gradient, reviewed by (Morisato and Anderson, 1995). Dorsal, an NF- κ B family transcription factor, translocates from the cytoplasm and accumulates in cellular blastoderm nuclei. Nuclei on the ventral side acquire high concentrations while dorsolateral nuclei acquire lower amounts (Steward *et al.*, 1988; Roth *et al.*, 1989; Rushlow *et al.*, 1989). The polarity and shape of the Dorsal gradient is determined by an extracellular signal originating on the ventral side of the perivitelline space (PVS). Generation of

this signal has been proposed to involve a protease cascade leading to the processing and activation of a 'ventralizing' or ventral specifying ligand (DeLotto and Spierer, 1986; Chasen and Anderson, 1989; Morisato and Anderson, 1994; DeLotto and DeLotto, 1998). How this process is spatially and temporally regulated and precisely where and when a gradient of any component or activity first appears is presently unknown.

Asymmetric positional cues for embryonic dorsal-ventral polarity are laid down during oogenesis and require the genes *gurken*, *torpedo* and *fs(1)K10* (Ray and Schupbach, 1996). The products of these genes function on the dorsal side of the developing oocyte to suppress ventral cell fate in the somatic follicle cells. The genes *nudel*, *pipe* and *windbeutel* are required in the follicle cells that secrete the egg shell and the vitelline membrane.

Maternal effect genes including *snake* (*snk*), *easter* (*ea*), *gastrulation defective* (*gd*) and *spatzle* (*spz*), are required in the germ line and their products are secreted into the PVS (Roth, 1994). *gd*, *snk* and *ea* encode serine proteases, which function in a protease cascade, and *spz* encodes a precursor of an NGF-like molecule, which is generally thought to be the ventralizing ligand (DeLotto and Spierer, 1986; Chasen and Anderson, 1989; Morisato and Anderson, 1994; DeLotto and DeLotto, 1998; Konrad *et al.*, 1998; Han *et al.*, 2000; Dissing *et al.*, 2001). GD functions before Snake, making GD the earliest known germline component and therefore a candidate for a protease which could directly interpret the ventral prepatter (Smith and DeLotto, 1994; Dissing *et al.*, 2001). Here, I address the role of GD in orienting and shaping the Dorsal gradient.

⁺Corresponding author. Tel: +45 35 32 21 06; Fax: +45 35 32 21 13; E-mail: rdelotto@biobase.dk

Fig. 1. (A) A GD structural model. The serine protease homology domain is lightly stippled and the positions of the active site residues (H, D, S) are indicated. The arrow indicates the position of the RK residue pair and the von Willebrand type A homology motif is in heavily stippled, while the predicted signal peptide is hatched. Expected molecular weights of cleaved fragments are indicated below. (B) A protein sequence comparison of GD and type A von Willebrand factor motifs. The arrow indicates the position of the activation site in murine complement factors C2 and B

Table 1. Phenotypic rescue of embryos from gd^p/gd^p females by microinjection with wt gd RNA

RNA concentration ($\mu\text{g/ml}$)	#emb	No cuticle (%)	Dorsalized (%)	filzkörper (%)	Ventral denticles (%)	Hatching (%)
Uninjected	100	3	97	0	0	0
1	105	49	23	19	9	0
10	139	38	6	16	40	0
100	127	52	2	2	44	0
1000	81	68	0	1	31	0

RESULTS

Gastrulation defective has structural similarities to the mammalian complement factors C2/B

The predicted protein encoded by gd was compared with members of the serine protease superfamily and it lacks sequence conservation within the activation peptide cleavage site. Examination of the protein data bank revealed two serine proteases, which also deviate from the consensus within the activation peptide region. The complement factors C2 and B are activated by cleavage between an arginine and lysine residue and ~12 amino acids after the cleavage site in factor C2 and B, a type A von Willebrand repeat motif is found (reviewed in Arlaud *et al.*, 1998). The gd amino acid sequence was compared with factors C2 and B and an alignment revealed that GD possesses an arginine lysine pair as well as a type A von Willebrand motif in a conserved geometry (Figure 1A). These features suggest that gd encodes a serine protease zymogen, which is

structurally similar to factors C2 and B, and is therefore activated by cleavage between Arg¹²⁸ and Lys¹²⁹. Site-directed mutagenesis studies and the analysis of interallelic complementation at gd are consistent with this model (Ponomareff *et al.*, 2001).

Phenotypic rescue of gastrulation defective by microinjection of gd SP6 RNA transcripts

To determine whether wild-type (wt) gd transcripts can rescue embryos from gd^- mothers, gd RNA at different concentrations was microinjected into embryos lacking gd function. As shown in Table 1, injection of wt gd RNA restores ventrolateral pattern elements such as filzkörper or ventral denticles, in a concentration-dependent manner. While no ventrolateral pattern elements were observed in uninjected embryos, when injected with 1 $\mu\text{g/ml}$ RNA, 19% of embryos exhibited filzkörper. At 100 $\mu\text{g/ml}$ RNA, 2% of embryos exhibited filzkörper and 44% exhibited ventral denticles. At 1 mg/ml, 31% of the embryos exhibited ventral denticles and the number of embryos with no cuticle increased

Fig. 2. (A) A dorsalized cuticle from a *gd*⁰/*gd*⁰ mother. (B) An embryo from a *gd*⁰/*gd*⁰ mother that was injected in the posterior pole with wt *gd* SP6 RNA at 10 µg/ml. (C) An embryo injected as in (B) but showing split ventral denticles. (D) Complete phenotypic rescue of an embryo from a *snk*²²⁹/*snk*²²⁹ mother by injection of wt *snake* SP6 RNA at the posterior pole.

to 68%. The increase in the no-cuticle phenotype is consistent with high concentrations of *gd* RNA specifying mesodermal cell fate, which does not make cuticle.

The phenotype of *gd* rescued embryos differs from *snk* RNA and *ea* RNA rescued embryos. Microinjection of *snk* and *ea* RNA can rescue completely the phenotype of embryos from *snk*- and *ea*-mothers and normalize d-v cell fates, producing hatching larvae (Figure 2D). However, in no case has ventralization been observed for high concentrations of wt *snake* or *ea* RNA (Chasen and Anderson, 1989; Smith *et al.*, 1994). While *gd* RNA restores ventrolateral pattern elements in a concentration-dependent manner the pattern was never normalized (*N* > 2000). Furthermore, while *snk* and *ea* RNA rescue shows no position dependence, the rescue observed with *gd* RNA is largely localized to the injection site (Figure 2B). Embryos injected with moderate RNA concentrations (*n* = 127) produce ventrally open cuticles with split ventral denticles near the site of injection (Figure 2C).

gd RNA ventralizes by expanding mesodermal cell fate without altering the slope of the Dorsal gradient

To determine whether microinjection of *gd* RNA transcripts could alter d-v cell fates in wt embryos, *gd* RNA was injected into OregonR embryos and the pattern was analyzed. After injection both the gastrulation pattern and larval cuticles were locally ventralized and all injected embryos failed to hatch. To determine how *gd* RNA microinjection alters the fate map, embryos were injected with *gd* RNA, and then either probed with antibodies against Twist, a mesodermal cell fate marker, or hybridized *in situ* for *rhomboid*, a ventrolateral cell fate marker (Thisse *et al.*, 1988; Arlaud *et al.*, 1998). In wt embryos, Twist is expressed as ventral stripe, 16–18 cells wide along

the anterior-posterior length of the blastoderm in the future mesoderm. The first domain of *rhomboid* expression in wt embryos is two ventrolateral stripes between 6 and 8 cells wide, within the neuroectodermal primordium. Since early lateral *rhomboid* expression is limited to a defined intermediate concentration of Dorsal, it has been used as a sensitive indicator of the slope and position of the Dorsal gradient (Roth, 1993).

To determine how *gd* RNA concentration affects the Dorsal gradient, RNA was injected at 50 µg/ml at the posterior of embryos from *gd*⁰/*gd*⁰ or wt females. Embryos were allowed to develop and *in situ* hybridized with *rhomboid*. Injected wt embryos (*n* = 145) exhibit two ventrolateral stripes, however they are shifted dorsally near the site of injection (Figure 3B). The width of the *rho* stripe is identical to that of wt embryos. Figure 4 illustrates a similar injection analyzed by expression of Twist. Near the site of injection, Twist expression is expanded dorsally whereas at the anterior it is closer to normal (Figure 4B). Thus, increasing GD dosage in wt embryos locally expands the mesodermal anlagen without altering the slope of the Dorsal gradient.

gd RNA transcripts were injected into an embryo from a *gd*⁰/*gd*⁰ mother (Figure 3C–E). Uninjected *gd*⁰/*gd*⁰ embryos show no lateral *rhomboid* expression at cellular blastoderm. Over the dorsal and ventrolateral regions of the *gd* injected embryo, *rho* is expressed as a uniform stripe approximately six cells wide, shifted dorsally near the site of injection. However, away from the injection site on the ventral side, the stripe broadens to cover a large part of the ventral region. Figure 3C and D illustrate the degree of variation seen in one injection series (*n* = 122). As before, the primary consequence of increasing *gd* concentration is to shift the position of the boundary between mesoderm and ventral neurogenic ectoderm dorsally, while the width of the ventral neurogenic ectodermal primordium remains constant. However, the ventral part of the egg responds more to an equivalent *gd* RNA concentration than dorsal or ventrolateral

Fig. 3. Effects of *gd* RNA injection upon *rhomboid* expression. (A) *In situ* expression of *rhomboid* in a wt embryo. (B) *rhomboid* in a wt embryo injected at the posterior pole with wt *gd* SP6 RNA. (C) *rhomboid* expression in an embryo from a *gd*⁰/*gd*⁰ mother, similarly injected (D) and (E). Lateral and ventral views of *rhomboid* in an embryo from a *gd*⁰/*gd*⁰ female injected with *gd* RNA showing expansion of *rhomboid* expression in a ventral zone. Orientation: anterior left, dorsal up.

Fig. 4. (A) Anti-Twist staining of a wt OregonR embryo. (B) Anti-Twist staining of an OregonR embryo injected at the posterior pole with wt *gd* SP6 RNA. Orientation: anterior left, dorsal up.

High *gd* RNA concentrations produce an ectopic dorsal-ventral axis in embryos from *pipe* null females

Pipe is a candidate for defining an asymmetric cue directing d-v pattern during embryogenesis (Sen *et al.*, 1998). To test whether *gd* RNA ventralization requires *pipe*, *gd* RNA was injected into embryos from *pip*⁶⁶⁴/*pip*⁶⁶⁴ females (Figure 5). At low *gd* RNA concentrations, no effect was seen. However, at 100 µg/ml, small ectopic patches of ventral denticles were observed (data not shown). Between 100–500 µg/ml localized expression of Twist appeared close to the injection site (Figure 5A). When hybridized *in situ* for *rho*, either one or two radial stripes spanning the d-v axis were generated (Figure 5B). In wt embryos a third *rho* stripe appears in the future amnioserosa very shortly after the two lateral stripes appear (Arlaud *et al.*, 1998; Roth, 1993). When two *rho* stripes were observed in embryos from *pipe*-null mothers, the distance between them was the same as the distance between the lateral *rho* stripes and the amnioserosal *rho* stripe. All injected embryos stained for Twist (*n* > 100) show only one patch of Twist expression near the injection site. When it appears, the second *rho* stripe correlates with amnioserosal *rho* expression. I conclude that high *gd* RNA concentrations in a *pipe*-null background induces an ectopic axis with the ventral pole defined by the point of injection. Embryos from, *snk*²²⁹-,

parts of the embryo as *rho* expands. In all injected embryos, the transition from stripe to broad expression is sharp and at the same d-v position. This increased response on the ventral side defines a previously unidentified stripe-like ventral zone.

Fig. 5. Orientation of the d-v axis by *gd* RNA in embryos from *pip⁶⁶⁴/pip⁶⁶⁴* mothers. **(A)** anti-Twist antibody staining. **(B)** *rhomboid* *in situ* hybridization. RNA was injected at the posterior pole in both embryos.

ea¹- and *spz¹⁹⁷*-null females were microinjected with 100 µg/ml *gd* RNA and remained dorsalized indicating that this effect requires their activities (data not shown).

DISCUSSION

The data presented here suggest that the shape of the Dorsal gradient is not directly determined by asymmetric cues in the eggshell but rather arises within the perivitelline space as a consequence of self-regulatory properties of the protease cascade triggered by GD (Dissing *et al.*, 2001). Localized phenotypic rescue is consistent with the idea that GD is membrane bound (Stein and Nüsslein-Volhard, 1992). Since GD can produce an ectopic axis in a *pipe*-null background, *pipe* activity is not required for binding. Binding of GD to a surface within the PVS is therefore independent of the spatial control of activation which is normally ventrally restricted and requires Pipe.

The spatial activation of GD must be regulated subsequently to and independently of binding. This could be explained via interaction of GD with a Pipe-modified proteoglycan. Since *pipe* is an heparan sulfate 2-O-sulfotransferase and its expression is restricted to the ventral third of the somatic follicle cell, it presumably modifies a somatically expressed proteoglycan (Sen *et al.*, 1998). Ventrally restricted modification by Pipe of this proteoglycan could control GD activation. It is known that complement and coagulation pathways can be both positively and negatively regulated by interaction with heparan sulfate proteoglycans (Selleck, 2000).

Since direct quantitative measurement of the Dorsal gradient is difficult, I chose to use one of the most sensitive markers for the position and slope of the Dorsal gradient, *rho* expression. The data suggest that a self-regulating patterning mechanism exists, which is independent of GD concentration and can determine the slope of the gradient. The injection phenotype is not exactly like that of embryos from females carrying mutations in *gurken* and *torpedo* (DER) (Roth and Schupbach, 1994; Roth *et*

al., 1995). In these mutations, while *rho* stripes are shifted dorsally, the mesoderm splits into two ventral furrows. This splitting has not been observed in any *gd* RNA injections.

An intriguing observation from these injections is that of a discontinuity along the width of the embryo. A ventral region appears to be more sensitive than lateral and dorsal parts of the embryo are to GD activity. While over most of the ventrolateral and dorsal surfaces a uniform width *rho* stripe was generated, away from the point of injection, *rho* was expressed in a broad ventral patch. The transition between stripe and broad zone is sharp, bilaterally symmetrical and always at the same relative position in injected embryos. These results suggest the existence of a ventral prepattern in the form of a broad 'stripe-like' zone with distinct boundaries.

Han *et al.* (2000), while also reporting ventralization by *gd* microinjection, indicated that microinjection of *gd* RNA into embryos from *gd^F/gd^F* mothers can also normalize and rescue some embryos to hatching. While this differs from my observations in *gd^d* and *gd^p*, it is possible that the ability to rescue *gd^F* is an allele-specific effect. *gd^F*, while an amorphic allele, results from a single amino acid change of Gly₄₄ to Asp in contrast to *gd^d* and *gd^p*, which are deletions and truncations, respectively. Allele-specific effects are observed at *gd* (Ponomareff *et al.*, 2001).

The RNA concentration required to produce an ectopic ventralizing signal in *pipe*-null embryos is significantly greater than that necessary to 'rescue' an embryo from a *gd*-null mother. In the *pipe*⁻ injections, I propose that high local concentrations of GD become proteolytically active at the ectopic site either by 'titrating out' regulatory serpins that normally control the cascade. Serpins specifically regulating this pathway have not yet been identified, but there is evidence for a serpin which binds Easter and evidence for a serpin regulating Spaetzle processing in innate immunity (Misra *et al.*, 1998; Levashina *et al.*, 1999). While the Dorsal gradient formed in injected *pipe* nulls is somewhat broader than in wt, it is still formed independently of Pipe. Therefore, the part of the pathway responsible for shaping the Dorsal gradient must have self-regulating properties which are independent of the ventral prepattern.

Speculation

The data suggest a model in which the Dorsal gradient is shaped dynamically via a proteolytic activation cascade during embryogenesis starting with a ventral stripe prepattern. The prepattern can be visualized as initially consisting of: (i) a broad ventral zone or stripe in which GD activity is potentiated, and (ii) the remainder of the PVS in which GD or the other proteases are inhibited. An interesting possibility is that the ventral stripe zone results from the modification of a uniformly distributed inhibitory substance through *pipe* post-translational modification. The sharp boundaries of the zone identified in these experiments suggests the ventral prepattern is not already graded. The fine-tuning of the slope of the Dorsal gradient may involve complex interactions within the proteolytic cascade downstream of GD or via feedback to GD. The results presented here are consistent with GD being a protease which has a pivotal role in interpreting the d-v prepattern as well as initiating and shaping the Dorsal gradient during embryogenesis.

METHODS

Isolation of full-length *gd* cDNA. A 0–2 h embryonic cDNA library (N. Brown, Cambridge) containing cDNA inserted after a *Xenopus* β -globin untranslated leader and an SP6 promoter was screened using a [γ - 32 P] end-labeled oligonucleotide (5'-CCATTGTCGGCAATGACG-3') derived from published *gd* cDNA sequence using standard methods. A full-length clone (*gd*cD7) was isolated and its primary DNA sequence was found to correspond to that described previously (Konrad *et al.*, 1998).

Synthesis of RNA transcripts and microinjection. Phenotypic rescue assays were conducted with eggs from homozygous females derived from the following stocks: OregonR (wt), *gd*9/FM7 and *ru st e pip664 ca/TM3;Sb. gd*cD7 was linearized at the 3' end by digestion with *Not*I and RNA was synthesized as described previously (Smith and DeLotto, 1994). RNA was injected into stage 2 embryos, which developed for 2 days at 20°C in a humid chamber, and cuticles prepared as described (Wieschaus and Nüsslein-Volhard, 1986).

Antibody staining and *in situ* hybridization. Rabbit anti-Twist antisera was a generous gift of Dr Maria Leptin. Stainings were conducted as described previously (Smith and DeLotto, 1994). cDNA for the synthesis of *rhomboid* probes (Arlaud *et al.*, 1998) was provided by S. Roth and *in situ* hybridizations were carried out as described (Roth, 1993).

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